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(54) Title: THE USE OF ANTIBODIES AGAINST CD48 FOR THE TREATMENT OF T AND B CELL LYMPHOMAS AND LEUKEMIAS

(57) Abstract

The present invention provides a method of treating T or B cell lymphomas or leukemias. The method involves administering to a subject an antibody of isotype IgG directed against CD48.

"THE USE OF ANTIBODIES AGAINST CD48 FOR THE TREATMENT OF T AND B CELL LYMPHOMAS AND LEUKEMIAS"

FIELD OF INVENTION

The present invention relates to a method of treating T and B cell lymphomas and leukemias. The method involves the use of antibodies targetted against CD48.

BACKGOUND OF INVENTION

Over the past decade, significant advances have been achieved in the chemotherapy of leukemias and lymphomas. Combination chemotherapy regimes and bone marrow transplantation (BMT) yield high rates of remission. However many of these remissions are not durable and the majority of patients diagnosed with hematopoietic neoplasms ultimately succumb to their disease. The long term survival of patients with non-Hodgkin's lymphoma and adult acute chronic leukemia are therefore still low. Currently, only 40% of patients with acute lymphoblastic leukemia (ALL) and intermediate or high grade non-Hodgkin's lymphoma achieve long term survival.

Simultaneous with theses advances in chemotherapy, Kohler and Milstein published their seminal report describing the production of monoclonal antibodies (MoAbs) by hybridomas. Because MoAbs can bind to antigens expressed on the surface of malignant hematopoietic cells, optimistic projections proclaimed that these agents could be used in serotherapy to specifically target and destroy those cells. Moreover, by offering distinct cytotoxic mechanisms, MoAb therapy could potentially circumvent tumour cell resistance. However, a number of major obstacles have limited the potential of this approach, including the problems of immunogenicity of rodent immunoglobulins, modulation of antigen by tumour cells, non-specific uptake of antibody by phagocytes, low binding affinity of some antibodies, and circulating antigen in the plasma in certain situations. While strategies have been proposed to deal with some of these problems, a further major difficulty is that most MoAbs lack the ability to kill cells after binding to the target surface antigen, either through activation of the human complement cascade, or through promotion of antibody-dependent cellular cytotoxicity (ADCC) mediated by cytotoxic T lymphocytes. This lack of cytolytic effector function in vitro has been

reflected in the generally disappointing results of intravenous antibody infusion studies in patients with leukemia and lymphoma (11).

CD48, a 47kd glycophosphatidylinositol-linked glycoprotein, has a number of characteristics which suggest it may be a good target for immunotherapy. CD48 is expressed on a wide range of lymphoid malignancies but not other tissues (2,3,4). Although both normal and malignant T and B cells express CD48, most CD34 positive cells do not express CD48. CD48 is also present at high levels on the surface of T and B cells. The biological function of CD48 in humans is still not clear. In mice CD48 is a high affinity ligand of CD2 (5) but is a low affinity ligand of human CD2 (6,7).

Anti-CD48 antibodies have been described (3), and one such antibody has been trialed for use in anti-tumour therapy (13). This trial involved the injection of up to 50mg of an IgM anti-CD48 antibody into four patients with Chronic Lymphatic Leukemia. However, only transient drops in circulating lymphocytes were observed. No impact on the progression of the underlying disease was observed in any patient. These results suggest that anti-CD48 antibodies do not provide strong anti-tumour effects.

The present inventors have now found that an antibody of appropriate isotype directed against the CD48 antigen has a strong anti-tumour effect in an animal model. The anti-tumour effect has been demonstrated in an animal model where treatment of mice containing tumour cells with an anti-CD48 antibody gave long term survival (cures) over those treated with a control antibody.

SUMMARY OF INVENTION

Accordingly, in a first aspect the present invention consists in a method of treating T or B cell lymphomas or leukemias which method includes administering to a subject in need thereof an antibody of class IgG directed against CD48.

The IgG isotype may be an isotype selected from mouse IgG2a, human IgG1, human IgG2 and rat IgG2b. When the subject is human, the preferred isotype is human IgG1 or IgG2. When the subject is murine, the preferred isotype is mouse IgG2a. When the subject is a rat, the preferred isotype is rat IgG2b.

When used herein the term 'antibody' refers to any specific binding substance having a binding domain with the required specificity. Thus, the

term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide including an immunoglobulin binding domain, whether natural or synthetic. Chimeric molecules including an immunoglobulin domain, or equivalent, fused to another polypeptide are therefore included.

In a preferred embodiment the antibody is directed against the HuLym-3 antigen which has recently been cloned and sequenced (9).

The present inventors have also found that Chimeric anti-CD48 antibodies, in which the Fc portion is replaced with a human Fc region, exhibit improved characteristics over murine anti-CD48 antibodies. These characteristics may include higher effector function, longer serum half-life and lower immunogenicity.

Accordingly, in a further preferred embodiment the antibody directed against CD48 is a chimeric (or humanised) antibody. The chimeric antibody may be a murine antibody in which at least part of the Fc portion is replaced with a human Fc portion.

It will be appreciated that the efficacy of the anti-CD48 antibody may be enhanced by conjugation to, for example, a radioisotope, cytokine, protein toxin or anti-cancer agent. The anti-cancer agent may be selected from doxorubicin, cisplatin, taxol, interferon, *Pseudomonas* exotoxin A, fumagillin, AGM-1470, tamoxifen, nitrosureas including ACNU, BCNU, CCNU and PCNU, diaziquone, decarbazine, hydrea, semustine, matulone, teniposide and terazante.

The method of the first aspect of the present invention may be used to treat non-Hodgkin's lymphoma or lymphoid leukemias.

One advantage of this invention resides in the finding by the present inventors that CD48 is expressed on less than 5% of CD34 positive progenitor cells. Thus, anti-CD48 antibodies should target lymphocytic leukemia and lymphoma cells whilst leaving the majority of CD34+ progenitor cells to expand and repopulate depleted cell types.

DETAILED DESCRIPTION OF INVENTION

In order that the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples in which:-

Figure 1. Internalisation of anti-CD48 antibody HuLy-m3.

Figure 2(a) and (b). Survival post CD48 therapy in SCID/Raji (kaplan Meier curves). SCID mice were injected with either (a) 1 x 10^6 Raji cells or (b) 5 x 10^4 Raji cells. Groups of five mice were injected with either 200ug of murine anti-CD48 antibody, HuLym3 or an isotype control antibody on days 0, 2 and 4 after Raji cell injection .

Figure 2(c). Effect of different anti-CD48 antibody doses. SCID mice were injected with 1 x 10^5 Raji cells and groups of five mice injected with either 200 Tg of isotype control, 200 Tg or 20 Tg IgG2a anti-CD48 antibody HuLym3 on days 0, 2 and 4 after Raji cell injection.

Figure 2(d). Effect of different anti-CD48 antibodies. SCID mice were injected with 5 x 10^5 Raji cells and groups of five mice injected with either 200 Tg of IgG2a isotype control, 200 Tg IgG2a anti-CD48 antibody HuLym3 or 200 Tg IgM anti-CD48 antibody on days 0, 2 and 4 after Raji cell injection.

Figure 3. ADCC assay of chimeric HuLym3, murine HuLym3 and rat Campath antibodies using the Raji cell line as target cells and human PBMC effector cells. Results shown are for an antibody concentration of 1ug/ml and an effector to target ratio of 25:1.

Materials and Methods

Cell Lines

The HuLym3 hybridoma cell line was a gift from Dr. Mauro S Sandrin, The Austin Research Institute, Melbourne (17) and was cultured in RPMI1640 with either 10% foetal bovine serum (FBS) or 10% bovine IgG free serum (Starrate), 2 mM glutamine at 37°C in a 37° incubator. The cell lines COS1, CHO-K1, Raji, Daudi, MOLT-4, U-937 and CCRF-CEM where obtained from ATCC. COS and CHO cells where cultured in 1:1 DMEM/F12 (CSL) with 10% FBS at 37°C, 5% CO2. Raji, Daudi, MOLT-4, U-937 and CCRF-CEM where cultured in RPMI 1640, 2mM glutamine, 10% FCS at 37°C and 5% CO2.

Production and purification of antibodies

Antibody (IgG2a) was purified from HuLy-M3 hybridoma conditioned media or from ascites fluid produced in either nude mice or BalbC/CBA crosses using protein A affinity chromatography (Pharmacia). The purity of the antibody was confirmed by 10% SDS-PAGE and the activity confirmed by flow cytometry using human leukemic cell lines. For animal experiments the antibody was further purified by ion exchange chromatography and gel filtration. An IgG2a isotype control antibody (anti-human TSH) was obtained from Bioquest and repurified as above. Protein concentration was estimated by absorbance at 280nm (18). Rat Campath-1 antibody was a gift from Bob Hale, MRC, Cambridge, UK. The IgM anti-CD48 antibody WM63 was obtained from Ken Bradstock, Department of Haematology, Westmead Hospital.

CD34/CD48 expression by Flow Cytometric Analysis of Human Mononuclear Cells.

Human peripheral blood (10 ml from patients and 50ml from normals) was obtained from normal individuals or CLL and NHL patients of St Vincent's Hospital, Sydney, Australia and was collected into EDTA tubes to prevent clotting and lymphocytes and monocytes (PBMC) prepared on a Ficoll gradient. Viable cells were counted by Trypan-Blue exclusion and resuspended to a final concentration, in PBS, of 1 x 10^6 cells/ml. For flow cytometric analysis anti-CD34-FITC, anti-CD45-PerCP and biotinylated anti-CD48 or isotype control antibodies were incubated with 5.0 x 10^6 cells for 30 minutes at 4°C washed once with 2 ml of wash media (PBS/1% BSA), streptavidin-PE was then added for 30 minutes at 4°C. Cells were washed twice, fixed in 500 μl of fixing buffer containing 1% sucrose and 0.5% paraformaldehyde in PBS and if not profiling immediately, stored at 4°C with the addition of 1 ml of PBS/1% BSA. Fluorescence was estimated within 24 h of sample preparation on a Coulter Epics flow cytometer.

Estimation of the number of CD48 molecules per cell.

Human peripheral blood mononuclear cells were prepared as described above and resuspended to a final concentration of 20 x 10^6/ml.

Simply Cellular Microbeads where used to estimate the number of binding sites per cell as per the manufacturers instructions.

Antibody Internalization

Examination of the internalization of CD48 monoclonal antibodies was performed by flow cytometry (19) and confocal microscopy. Human peripheral blood mononuclear cells were isolated as described above. For flow cytometry 5-10 x 10^6 cells were incubated with excess anti-CD48 or control antibodies (10-20 ug / 0.5-1.0 x 10^6 cells) in phosphate-buffered saline (PBS) for 60 minutes at 4^0C. After washing, the cells were resuspended in complete medium (RPMI/10%FCS) and incubated for intervals at 37^0C in a 5% CO2 incubator. After the appropriate incubation time FITC conjugated anti-mouse or anti-human IgG1 antibody was added for 30 minutes at 4^0C. After washing with cold PBS/1% BSA twice, the cells were fixed with 0.5% paraformadehyde, 1% sucrose in PBS for 15 minutes at room temperature and resuspended in PBS/1% BSA before analysis by flow cytometry.

For confocal microscopy, human PBMC were prepared and incubated with test monoclonal antibody at 4^0C and/or 37^0C as described above. After washing, the cells suspensions were duplicated and fixed either with 4% paraformadehyde in PBS (cell surface stain) or 4% paraformadehyde/0.1% Triton X-100 in PBS (intracellular and surface stain) for 30 minutes at room temperature. After washing with PBS/1% BSA, the cells were incubated with FITC conjugated anti-mouse or anti-human IgG1 antibody for 30 minutes at 4^0C. Cells were then washed with PBS and the pellets were mixed with 150 ml of PPD (1mg/ml r-phenylenediamine in 90% (v/v) glycerol, 10% (v/v) PBS pH 8.0) as an anti-fading agent, and placed on a glass slide and covered with a circular cover slip. To examine Ab internalization, confocal laser scanning microscopy (CLSM) was performed using a Sarastro 2000 CLSM (Molecular Dynamics, Sunnyvale, CA, USA), with a plan apochromat 60x/1.40 NA oil immersion lens and an argon-ion class II laser. Optical sections (usually 0.3 mm intervals) through FITC-labelled cells were captured using a 50mm fixed pinhole, with excitation at 488 nm, a 510 nm beam splitter and a 510nm barrier filter. Image processing was performed using a Silicon Graphics Personal Iris 4D 35 workstation. The cell viability was examined by Trypan blue exclusion.

Therapy of SCID mice with Raji lymphoma by HLM3

Six to eight-week old female SCID mice (CB17) were commercially obtained from Animal Resources Centre, Western Australia. Mice were housed in a C1 Lab maintained as a specific pathogen-free (SPF) facility. Groups of five mice were injected intravenously with Raji cells in RPMI 1640 (200Tl) on day 0. Antibody in 200 Tl RPMI 1640 was then injected i.v at days 0, 2 and 4. Mice were observed and weighed daily and sacrificed on the onset of hind leg paralysis. Blood and tissue samples were taken for further analysis. Bone marrow collected from one femur and blood were analysed immediately and collected tissues were either frozen in liquid nitrogen or fixed in formalin/PBS. Bone marrow and blood were analysed by flow cytometry to determine the percentage of human cells. Anti-mouse CD45-PE (PharMingen) and anti-human CD45-FITC (Becton Dickinson) were used to detect mouse and human leukocytes by flow cytometry as described above. Collected tissues were sectioned for histological stain and immunohistochemistry. Histological stain was performed using Haematoxylin and Eosin stain For immunohistochemistry, the sections were incubated with normal rabbit serum for blocking the endogenous peroxidase and then, incubated with anti-CD20 (Dako) or HuLy-m3 for 2 hours at 37^{0}C. After washing, the sections were chased by rabbit anti-mouse Ig-HRP (Silenus) and counterstained with Haematoxylin and Scotts blue. After washing again, it was dehydrated and mounted in Eukitt.

Cloning of Variable Region Genes of HLM3

Total RNA was isolated from the HuLym3 hybridoma (19) and used for first strand cDNA synthesis using heavy or light chain 3'-primers from Ig-Primer Kit (Novagen) . For PCR amplification of the variable heavy and light chain regions, cDNA was subjected to 35 cycles of PCR in separate tubes with various 5'- primers (Ig - Prime Kit, Novagen). The VH and VL PCR products were cloned into T-vector (20) by electroporation and transformed into E coli (DH5a). All of the VH and VL PCR products were DNA sequenced using T7 Sequencing kit (Pharmacia Biotech) in both forward and reverse directions. At least two clones for each PCR product was sequenced to minimise PCR errors. The amino acid sequence of the heavy and light chains was determined by N - Terminal amino acid sequencing of the alkylated

seperated chains in a 477A Protein Sequencer & 120A Analyser (Applied Biosystems).

Construction of chimeric HLM3

The expression vectors, HCMV-Crl and HCMV-Ck (21) were gifts from Mary Bendig (Medical Research Council, Laboratory of Molecular Biology, UK). The VH and VL inserts were removed from these vectors by Hind III and Bam HI digestion. The leader sequence was obtained by digestion with Hind III and Apa I from the M13-VkPCR1 vector which was a gift from Greg Winter (Medical Research Council, Laboratory of Molecular Biology, UK). The VH and VL were re-PCR amplified to introduce a 5' Apa LI site to both variable regions and 3' Bam HI site and Bgl II site to VH and VL respectively. The Hind III - Apa LI leader sequence and Apa LI - Bam HI VH sequence or Apa LI - Bgl II VL sequence where ligated in a three component ligation into the Hind III - Bam HI digested HCMV vectors. The ligation products were transformed into E coli , DH5a by electroporation (2.2 kv, 250 uF capacitance using a Gene Pulser (BioRad).

Expression in COS1 and CHO-K1 cells

Chimeric antibody was expressed in COS and CHO-K1 cells. The DNA was introduced into the COS1 and CHO-K1 cells by electroporation using the Gene Pulser apparatus (270 volts, 250 mF) (BioRad). Transformants were cultured under G418 (0.4mg/ml) selection and high yielding clones screened using a sandwich ELISA specific for assembled human antibodies.

Chimeric HLM3 was purified from cell culture supernatant by using Protein A Sepharose CL-4B (Pharmacia) or POROS 50A affinity chromatography (Perfusion Chromatography) eluted with 100 mM glycine (pH 3.0), and dialysed against PBS (pH 7.4) for buffer exchange. The concentration of stock solution is higher than 1mg/ml.

ELISA of Assembled Chimeric Antibody Expression

A Sandwich ELISA was used for determining the concentration of assembled human antibody. Goat anti-human IgG Fc specific antibody (Jackson ImmunoResearch Laboratories, Inc., USA) was coated on to Nunc Maxisorb plates at 2ug/ml at 4oC overnight. Anti-human kappa light chain - Biotin (The Binding Site Ltd, England) was used as a chase antibody

(0.5ug/ml) followed by alkaline phosphatase conjugated egg white - avidin (Jackson) (0.5ug/ml) and 1 mg/ml of phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma) in carbonate buffer were added into the plates in succession with washing between steps. The absorbance read at OD 405nm on ELISA Reader (Dynatech MR7000, Baxter). The human IgG (Jackson ImmunoResearch Laboratories, Inc., USA) was used as a standard (0.25 to 1000ng/ml). All antibody incubations where at least 1 hour at 37^0C.

ADCC Assay

ADCC was determined using a Cytotoxicity Detection Kit (LDH) (Boehringer Mannheim). Peripheral blood mononuclear cells (PBMC) were isolated from a healthy donors and used as effector cells. The human Raji cell line was used as a source of target cells. Campath1 (CDw52) monoclonal antibody (rat IgG2b) was used as a positive control. Mouse IgG2a (anti-TNP, Pharmagen) and human IgG1 (The Binding Site) antibodies were used as isotype controls for HuLym3 and chimeric HuLym3, respectively. PBMC where resuspended at 1 x 10^7/ml in assay medium (RPMI1640 with 1%BSA). Raji cells were adjusted to a final concentration of 1 x 10^5/ml in assay medium and incubated with different concentrations of each antibody (0.01-10ug/ml) for 30 minutes at RT. The effectors and Ab labelled target cells (1 x 10^5) were added at different ratios and incubated for 4 hours at 37^0C , 5%CO2. Released lactate dehydrogenase (LDH) activity was determined using the cytotoxicity detection kit as per the manufacturers instructions. The average absorbance of the triplicates was calculated and the background (assay medium only) corrected and then, substituted into the following equation:

$$\text{Specific Lysis (\%)} = \frac{\text{(E+T-Ab) - (E+T only) - low control}}{\text{high control - low control}} \times 100$$

Low control: target cells in assay medium
High control:target cells and 2% Triton X -100
E+T-Ab:effectors and target cells labelled with Ab

E+T:effectors and target cells only

Results

Characterisation of CD48 antigen.
Estimation of CD34+/CD48+ cells

The percentage of CD34+ cells expressing CD48 was determined by triple label flow cytometry using antibodies against CD34, CD45 and CD48. CD34+ cells were initially selected then CD48+/CD45+ cells were examined. Two healthy donor's and two CLL and NHL patient's PBMC were examined. Less than 5% of CD34 positive cells in healthy individuals and patients with CLL and NHL were found to express CD48. All samples examined had a level of around 4% ($\pm$ 1%) of CD34+ cells being CD48+.

Number of anti-CD48 binding sites/cell

The number of anti-CD48 binding sites per cell was estimated using Quantum Simply Cellular Microbeads and flow cytometry. Healthy individuals (two) and CLL patients (two) had similar numbers of binding sites with approximately 40000 $\pm$ 10000 binding sites present per cell. The B-lymphoma cell line Raji, expressed higher levels of CD48 than patient or normal individuals with approximately 200000 binding sites/cell.

Internalization of CD48 monoclonal antibody

Antibody internalisation was examined by two methods:- i) the loss of surface labelled antibody was monitored by flow cytometry and ii) confocal microscopy was used to determine the location (surface or intracellular) of labelled antibody. The anti-CD3 antibody, OKT3, which is known to readily internalise into cells was used as a positive control (22). The results are shown in Figure 1. Figure 1 shows a rapid signal loss from cells labelled with OKT3 and incubated at 37°C with the signal dropping to half initial value after approximately 30 minutes. In contrast, cells labelled with anti-CD48 antibody lost no signal after 24 hours. Experiments performed on PBMCs isolated from healthy individuals, CLL patients and leukemic cell lines all gave similar results.

Establishment of a B cell lymphoma model in SCID mice

To determine if antibodies against CD48 could mediate an anti-tumour effect in vivo we established a model of B cell lymphoma in SCID mice. All untreated SCID mice injected intravenously with the human Raji B-cell lymphoma cell line developed hind leg paralysis. The on set of paralysis was reproducible and depended on the injected cell dose. SCID mice injected with 1×10^6 or 5×10^4 cells developed paralysis after 19 ± 2 days or 34 ± 3 days respectively. Mice were sacrificed at the onset of paralysis. On autopsy mice were found to have disseminated tumour in various organs including liver, kidney, ovary, spleen, lung, lymph node. Grossly the neoplastic infiltrates frequently formed distinct, white, variably sized nodules. Immunohistochemisrty confirmed the presence of distinct nodules of tumour cells. These cells stained positive with antibodies specific for human CD20 and CD48. Bone marrow from the femur of sacrificed mice contained 20 - 60% human cells but no human cells where ever detected in the peripheral blood.

Inhibition of tumour cell growth in SCID mice by anti-CD48 antibodies

Groups of five SCID mice injected with Raji cells on day 0 were treated with 200ug murine anti-CD48 antibody or an isotype control antibody on days 0, 2 and 4. The results are shown in Figures 2(a) and (b). Treatment of mice injected with 1×10^6 Raji cells with anti-CD48 antibody resulted in a 40% increase in the time to hind leg paralysis. 4 of 5 mice injected with a cell dose of 5×10^4 cells and treated with the same dose acheived long term survival. Those treated with an isotype control antibody developed hind leg paralysis after approximately 32 days. The effect of anti-CD48 antibody dose on mouse survival was also investigated. Figure 2c indicates that an antibody dose of 20 Tg can prolong the time to hind leg paralysis in 60% of mice. Figure 2d shows the anti-tumour effect of anti-CD48 antibodies of different isotypes. An IgM antibody shows no increased survival of mice over the control antibody while the IgG2a antibody shows a marked survival benefit. These results indicate the murine anti-CD48 murine antibody HuLym3 can mediate a strong anti-tumour effect in vivo.

Cloning of The HLM3 Variable Region Genes

Based on the strong in vivo anti-tumour activity we cloned the variable regions of this anti-CD48 antibody for the construction of a mouse/human chimeric antibody. The variable regions where amplified by PCR using degenerate primers (Ig-Primer Kit, Novagen, USA). Use of these primers resulted in the generation of multiple PCR products. Two separate products for the Vh and three individual products for the Vl. The correct Vl product was determined by comparison of the predicted amino acid sequence with the determined N-terminal amino acid sequence and confirmed by data base searches. The Vh product was identified by Kabat data base searching which indicated that one product contained a 50bp deletion. Amino acid sequence could not be obtained from the heavy chain presumably due to the N-terminus being blocked.

Construction of Chimeric HLM3 and the Expression in CHO Cells

The anti-CD48 Vh and Vl gene segments were used to construct chimeric antibody expression vectors, HCMV.Vh and HCMV.Vl. Plasmid DNA was cotransfected into COS cells for transient expression and CHOK1 cells for stable expression. CHOK1 clones expressing the highest levels of chimeric antibody were picked after G418 selection using a sandwich ELISA The structure of the chimeric antibody was confirmed by SDS-PAGE and western blots (23). The chimeric antibody was shown to have near identical binding properties to the murine version when tested on various cell lines and PBMC samples (23) and to have the same relative affinity as the murine version as determined by a competitive ELISA (23).

ADCC of mHLM3 and cHLM3

The chimeric antibody was able to mediate significant lysis in ADCC assays using human PMBCs as effector cells. Concentrations of the antibody ranging from 0.01-10 ug/ml were tested at effector to target cell ratios of 6:1, 12:1, 25:1, 50:1 and 100:1. The rat Campath-1 antibody (IgG2b) was also included in some assays. Chimeric HuLym3 mediated specific lysis 2-6 times more efficiently than did the murine HuLym3 and 2-3 times higher than rat Campath1. The level of specific lysis with chimeric HuLym3 was similar for antibody concentrations between 0.1 and 5ug/ml and effector to target ratios of 12:1 to 50:1 with chimeric HuLym3 typically giving over 60%

specific lysis. Results of a typical ADCC assay are shown in Table 1 and Figure 3. The observed maximal specific cell lysis was dependent on the source of the effector cells as previously described (24).

Table 1

Donor	Effector	Target cell	Antibody	ug/ml	E:T	Max. Lysis %
M. 45yr	PBMC	Raji	Campath-1G	1.0	25:1	49.0
			mHLM3	1.0	25:1	32.9
			cHLM3	1.0	25:1	73.2

Discussion

CD48 is expressed on the surface lymphocytes, monocytes and the vast majority of lymphocytic leukemia and lymphoma cells (2,3). To evaluate the potential of targeting CD48 for treatment of lymphoma and leukemia we have further characterised a number of properties of CD48. CD34 represents an early marker in haemopoietic cell development (25). It would be beneficial for a therapeutic to target leukemia or lymphoma cells but not the CD34+ cells. We have shown that antibodies against CD48 will not target most CD34+ cells, as CD48 is expressed on less than 5% of CD34+ cells. Thus if all CD48+ cells were removed by antibody treatment the remaining CD34+ progenitor cells could expand and repopulate the depleted cell types.

Crosslinking by a bivalent antibody can result in patching, capping, and finially internalisation of surface antigen. The rates at which this process can occur vary from antigen to antigen. Some lymphoid differentiation antigens such as CD3 and CD7 can be internalised in minutes, whereas for others such as CD45 and CAMPATH-1 the process may take hours. Anti-CD48 antibodies remain present on the surface of cells for at least 24 hours without modulation. The stability of anti-CD48 antibodies on

the surface of cells is likely to play an important role in the observed cytotoxic effects mediated by this antibody.

There does not seem to be any significant difference in the expression level of CD48 on the surface of PBMC from healthy and CLL patient donors. Both sources of cells bound antibody at about 40000 sites/cell . CD48 is induced to high levels of expression on EBV-infected B cells (26) This was confirmed by analysis of the human Raji cell line which is EBV+ and expresses approximatly 200000 sites/cell.

An IgM anti-CD48 antibody has been used in a pilot phase I clinical trial (13). Four CLL patients were treated with up to 64mg of antibody over 6 days. A significant but transient reduction in the number of circulating leucocytes was observed. The IgM anti-CD48 antibody was capable of strong complement activation in-vitro but similar to the IgM Campath antibody which was capable of massive complement activation only caused transient reductions in circulating tumour cells. Further clinical trials with different isotypes of the Campath-1 antibody suggested that mediation of ADCC was important for a strong anti-tumour effect in-vivo (14, 15). Murine IgG2a and human IgG1 antibodies have been shown to mediate ADCC and anti-tumour activity (27).

We have shown that an anti-CD48 antibody can medite a potent in-vivo anti-tumour effect and can give long term survival to SCID mice injected with Raji cells, which if treated with an isotype control antibody develop hind leg paralysis after approximately 34 days. The disease presentation in this model is similar to other SCID mouse models using human B-cell lines (28), such as the Daudi cell line (29). However, the time course to hind leg paralys is much more rapid with the Raji cell line in comparision with the other models. For example, $1x10^6$ Daudi cells injected IV into SCID mice results in hind leg paralysis after about 34 days while the injection 1×10^6 Raji cells resulted in hind leg paralysis after about 20 days. Thus the Raji cell line causes a more rapid form of disease. Thus we report cell dosing with Raji cells at 1×10^6, a similar cell dose as reported in other models, and the results of a lower cell dose which allows survival of the mice for approximately one month, a similar survival period for untreated animals as reported the Daudi SCID model. At the higher cell dose, treatment with anti-CD48 antibody prolonged the time to hind leg paralysis by 40%. For the lower cell dose experiment where the mice developed hind leg paralysis after

32 days treatment with anti-CD48 antibodies produced long term survival in the majority of mice with mice surviving at least ten times longer than untreated animals. We have also shown that the antibody is active at much lower doses.

The therapeutic effect of the anti-CD48 antibody in SCID mice appears to be antigen specific as an isotype control antibody which does not bind to Raji cells has no therapeutic effect. The cytotoxic effect of the anti-CD48 antibody is likely to depend on a number of features including inability of the antibody to modulate the antigen and the effective interaction of murine IgG2a with murine effector functions. Because SCID mice lack significant T and B cell responses any effector response would either a direct toxicity of the monoclonal antibody on the tumour cells or the recruitment of other cells with cytotoxic activity such as neutrophils, macrophages or natural killer cells. As the anti-CD48 antibody has no direct cytotoxicity on Raji cells (unpublished results) the recruitment of effector cells seems the most likely explanation of the observed anti-tumour effect.

We have also attempted to address some of the problems associated with using murine monoclonal antibodies therapeutically by producing an antibody in which the Fc portion is replaced with a human Fc region. A chimeric antibody is predicted to have a number of improved characteristics for clinical use over the original murine antibody including higher effector function, longer serum half-life and lower immunogenicity (14, 15, 16). Chimeric antibodies consisting of a human IgG1 constant region have, in numerous cases exhibited a higher in-vitro cell killing activity in ADCC assays with human effector cells (14, 15). Chimeric antibodies usually have a half-life at least 5 fold longer than murine antibodies in humans (16) and being 75% human the immune response to chimeric antibodies is often much less than the murine equivalent (16).

Replacing the murine Fc with human Fc region may allow the acquisition of novel or enhanced effector functions in vitro and in vivo. This was clearly demonstrated in vitro where the chimeric antibody was able to mediate markedly enhanced lysis of CD48+ Raji cells using human effector cells in comparison to the murine version. Similar results have been found for a number of chimeric antibodies (30). We have trialed the murine version of the antibody in SCID mice as it would be predicted that the murine IgG2a

isotype of this antibody would be the most effective in mediating murine effector functions in mice (27).

Thus we have demonstrated that an antibody against CD48 is capable of mediating a strong anti-tumour effect in SCID mice and a chimeric version of this antibody is capable of mediating strong ADCC activity with human PBMC effector cells. These properties suggest anti-CD48 antibodies may be useful in the treatment of a number of diseases including lymopoid leukemias and lymphoma.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:

1. A method of treating T or B cell lymphoma or leukemia which method includes administering to a subject in need thereof an antibody of class IgG directed against CD48.

2. A method according to claim 1 in which the IgG isotype is selected from mouse IgG2a, human IgG1, human IgG2 and rat IgG2b.

3. A method according to claim 1 or claim 2 in which the antibody is directed against the HuLym-3 antigen.

4. A method according to any one of claims 1 to 3 in which the antibody is conjugated to a compound selected from a radioisotope, cytokine, protein toxin or anti-cancer agent.

5. A method according to any one of claims 1 to 4 in which the T or B cell lymphoma or leukemia is non-Hodgkin's lymphoma or lymphoid leukemia.

6. A method according to any one of claims 1 to 5 in which the antibody is a humanised antibody.

7. A method according to claim 6 in which the humanised antibody is a murine antibody in which the F_c region or part thereof is replaced with a human F_c region or part thereof.

8. A method according to any one of claims 1 to 5 in which the subject is a human and isotype is human IgG1 or human IgG2.

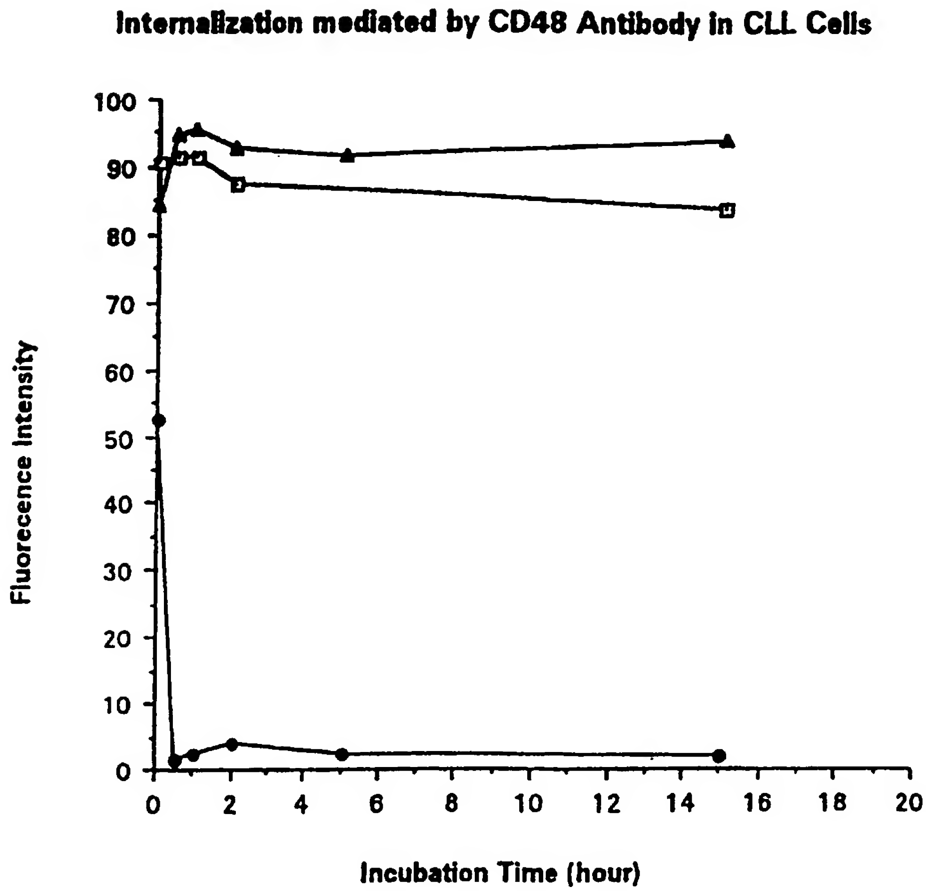
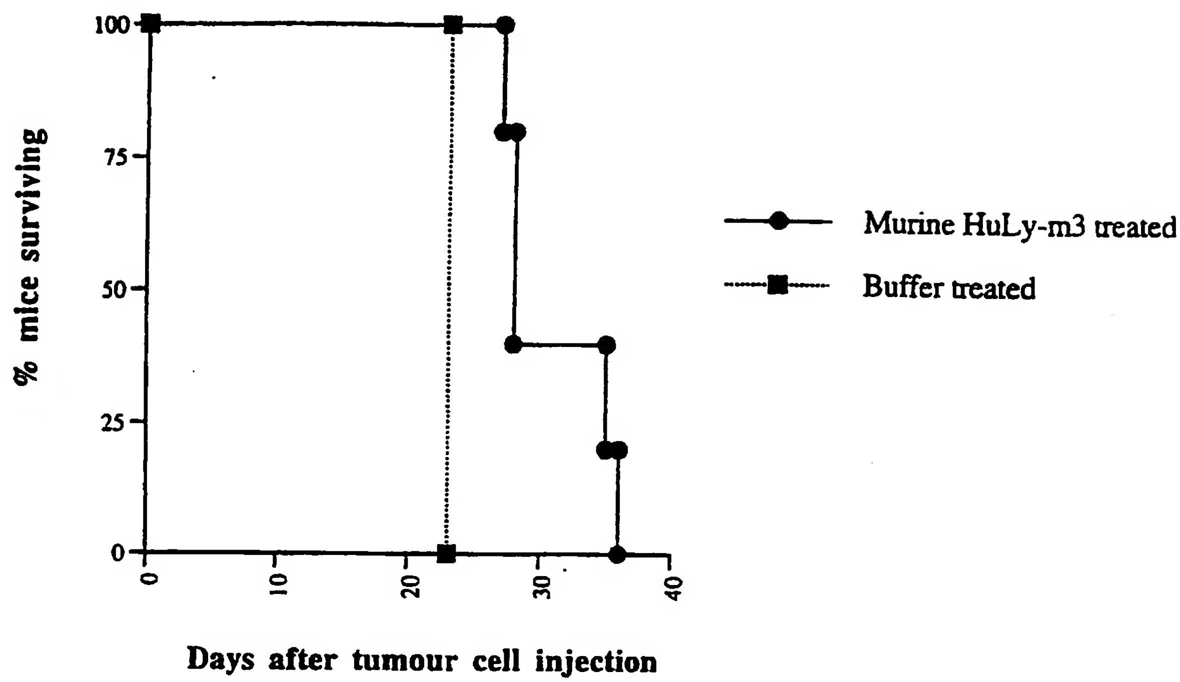

Internalization mediated by CD48 Antibody in CLL Cells
Fluorecence Intensity
0
10
20
30
40
50
60
70
80
90
100
0
2
4
6
8
10
12
14
16
18
20
Incubation Time (hour)

FIGURE 1



100
75
50
25
0
% mice surviving
0
10
20
30
40
Days after tumour cell injection
Murine HuLy-m3 treated
Buffer treated

FIGURE 2A

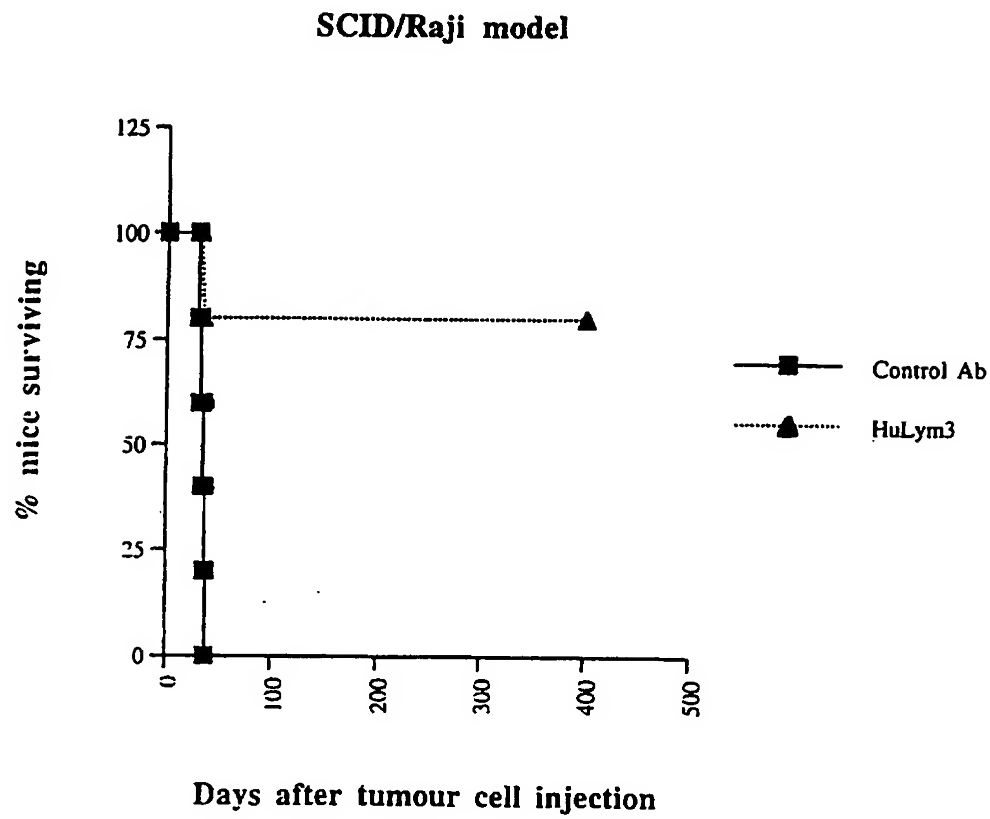

SCID/Raji model
% mice surviving
125
100
75
50
25
0
0
100
200
300
400
500
Days after tumour cell injection
Control Ab
HuLym3

FIGURE 2B

SCID/Raji model: Antibody Dose

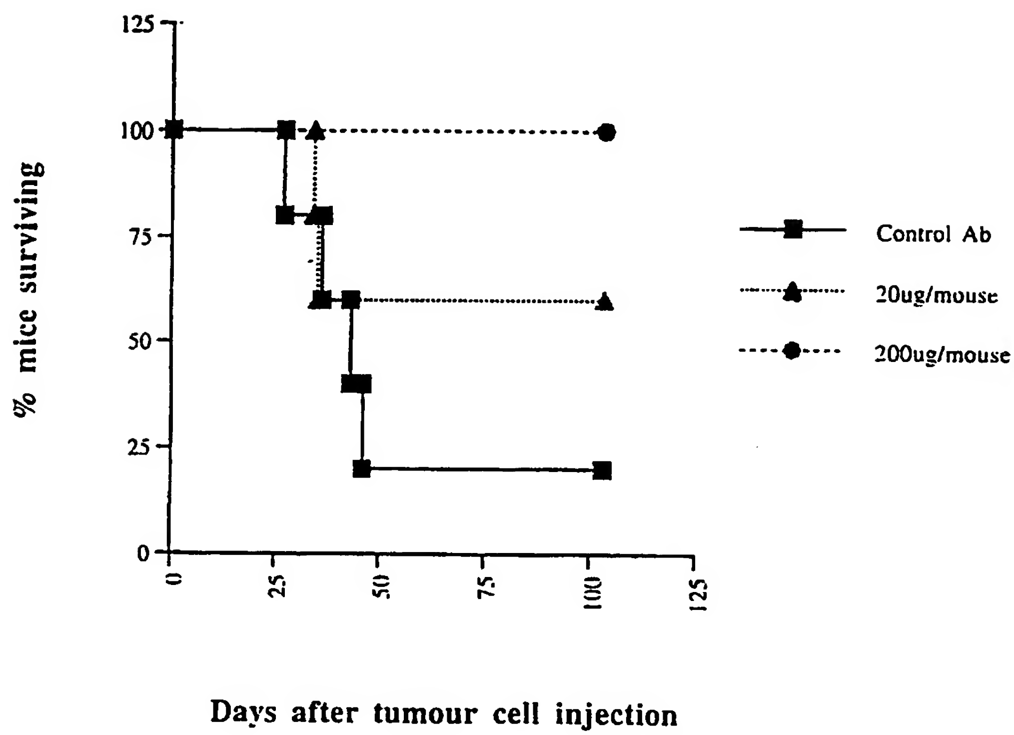


FIGURE 2C

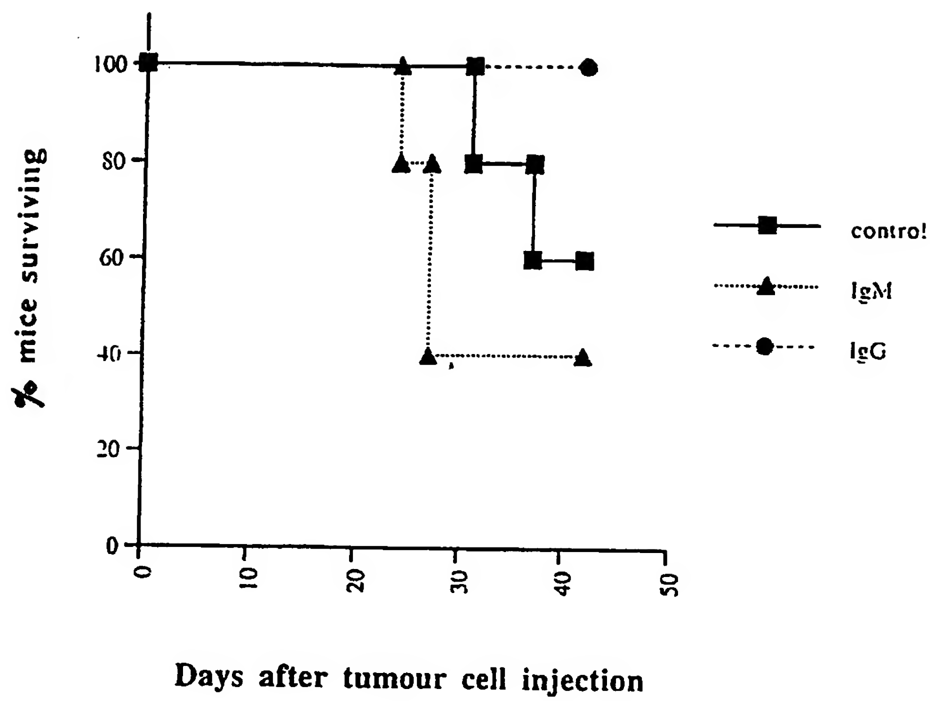

100
80
60
40
20
0
% mice surviving
0
10
20
30
40
50
Days after tumour cell injection
control
IgM
IgG

FIGURE 2D

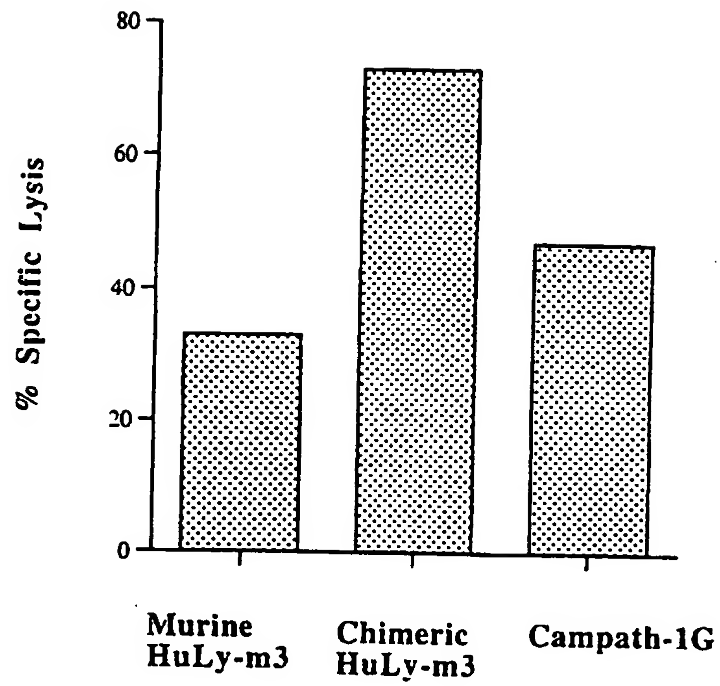

% Specific Lysis
80
60
40
20
0
Murine
HuLy-m3
Chimeric
HuLy-m3
Campath-1G

FIGURE 3

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00195

A. CLASSIFICATION OF SUBJECT-MATTER

Int Cl[6]: A61K 39/395, 39/44, 45/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: A61K 39/395, 39/44, 45/05

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT) [DC()48 OR CD48 OR HULYN () 3 OR HULYM 3 OR HULUM ()M3] and
) Keywords [ANTIBOD? OR IMMUNOGLOB?] and
CA) [Lymphoma or leukemia or neoplasm () inhibitors or Hodgkin () disease]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Blood (1995), Volume 86(4), Hertestein, et al., "Emergence of CD52 -, phosphatidylinositolglycan-anchor-deficient T lymphocytes after in vivo application of Campath - 1H for refractory B-cell non Hodgkin lymphoma" pages 1487-92 Whole document	1-5
A	Derwent WPAT Online Abstract Accession No. 94-269293, JP 06-269293 (SUMITOMO ELECTRIC IND LTD), 27 September 1994	1-5

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* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 1997	06 JUN 1997
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